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Qualitative and quantitative determination of biologically active low-molecular-mass thiols in human blood by reversed-phase high-performance liquid chromatography with photometry and fluorescence detection

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Abstract

The reversed-phase high-performance liquid chromatographic method employing photometry and fluorescence detection is described for the precise reproducible simultaneous measurement of total homocysteine (tHcy), cysteine (Cys), and glutathione (GSH) in human blood. Sample preparation involves conversion of disulfides to free thiols with triphenylphosphine, precipitation of proteins with trichloroacetic acid, conjugation of the thiols with monobromobimane (mBrB). The aminothiol assay is optimized by reduction and derivatization step conditions (pH, temperature and time of reactions) to obtain reliable quantitative results within the concentration range corresponding to normal and pathological levels of these thiols in human blood.

Keywords: Thiols; Aminothiols; Homocysteine; Monobromobimane; Cysteine; Glutathione

1. Introduction

Determination of homocysteine (Hcy), cysteine (Cys) and glutathione (GSH) in physiological liquids is important for the diagnostics of several human diseases, especially premature arteriosclerosis, occlusive vascular and neurodegenerative disorders, leukemia, diabetes, acquired immunodeficiency syndrome (AIDS), as well as in drug therapy monitoring.

The facile oxidation of aminothiols (ATs) results in a variety of disulfides in vivo. These include the low-molecular-mass compounds: cystine, cystinylbisglycine, homocystine and glutathione disulfide, and their corresponding mixed disulfides [1–4]. Sulfhydryl compounds also form mixed disulfides with proteins, and in plasma, a considerable fraction of these compounds are associated with albumin [1]. Although a number of the techniques were employed to assay these thiols, including HPLC with different detection (fluorimetric [1,2,5–7,9], photometric [12–14], electrochemical [8,10,15], GC [16] and radioenzymatic methods [1,17], the methods based on different ways of disulfide reduction and derivatization [1,2,5–9], some problems still arose associated with a complexity of the procedure and its quantitatation reproducibility, particularly in case of such multi-component samples as blood and urine.

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Simplification of the sample preparation, improving the sensitivity and reliability of the results is still being carried out. We developed a simple, sensitive and precise gradient RP-HPLC method for the identification and simultaneous quantitative determination of biologically active ATs, such as Hcy, Cys, and GSH. The method was based on disulfide reduction with triphenylphosphine (TP) and derivatization with monobromobimane (mBrB). The maximum yield of thiol adducts greatly depended on the pH of the derivatization step. The time and the temperature of reduction and derivatization reactions were also optimized. A narrow-bore HPLC system equipped with a UV detector was employed for the method optimization, routine analysis of bimane–thiol adducts (BTAs) and for rough plasma assays as well. An additional on-line fluorescence detection was applied for determining BTAs in complicated mixtures of compounds, which hampered UV detection or for ATs highly sensitive assays. A higher selectivity and sensitivity of the HPLC system equipped with the fluorometer promoted to quantify AT–mB conjugates more precisely and reliably in a wider AT concentration range. Due to the method simplicity and reliability, the developed procedure is suitable for routine analysis of tHcy and other biologically significant low-molecular-mass thiols in biological samples, required for clinical diagnostics and biochemical research.

2. Experimental

2.1. Chemicals and reagents

Ethylenendiaminetetraacetic acid disodium salt (EDTA), trichloroacetic acid (TCA), sodium citrate and dioxane were obtained from Sigma (St. Louis, MO, USA). Monobromobimane (Thiolyte) was from Calbiochem (San Diego, CA, USA). L-Cysteine (Cys), L-Cystine [$(Cys)_2$], DL-*meso*-homocystine [$(Hcy)_2$], reduced glutathione (GSH), oxidized glutathione (GSSG), sodium borohydride, triphenylphosphine, hydrochloric acid were purchased from Merck (E. Merck, Frankfurt, Germany), and trifluoroacetic acid (TFA) was from Perkin-Elmer (Warrington, UK). Acetonitrile was from Criochrom (St. Petersburg, Russia). Purified water from a Milli-Q system, Millipore (Molsheim, France) was used throughout the experiments. The liquids used for the HPLC systems were filtered through 0.22-μm membranes (Millipore) and degassed with helium for 3 min at 25°C. All solutions were mixed on a vibration mixer. Triphenylphosphine was prepared as 427.2 m*M* solution in water dioxane (80%, v/v) and 2 *M* hydrochloric acid (1%, v/v). Monobromobimane was prepared as 8.72 m*M* solution in water acetonitrile (9%, v/v) containing 2 m*M* sodium EDTA. The working solutions of 20 μmol/ml L-cysteine (L-Cys), reduced glutathione (GSH) and 10 μmol/ml L-cystine [$(Cys)_2$], DL-*meso*-homocystine [$(Hcy)_2$], oxidized glutathione (GSSG) in 0.2 *M* hydrochloric acid were prepared and kept at −20°C. Their aliquots were tested according to Section 2.4 omitting the protein precipitation step.

2.2. Instrumentation

HPLC analyses were performed on two systems. System 1 consisted of a narrow-bore high-performance liquid chromatograph Milichrom A-02 (Envirochrom A-02) (Chromatography Institute Eko-Nova, Novosibirsk, Russia) equipped with syringe pumps, a thermostated column compartment, an autosampler, the photometric cell 1.2 μl in volume, and the spectrophotometer monochromatic multiwavelength (190–360 nm) detector. System 2 included two pumps Model 110 A (Beckman Instruments, San Ramon, CA, USA), spectrofluorometer Model 121 (Gilson, Gambetta, France) with on-line spectrophotometer Model 100-40 (Hitachi, Japan), Controller Model 420 (Beckman Instruments), and an injector Model 210 (Beckman Instruments). The fluorescence detector output was recorded on Shimadzu Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan) and recorder Model 9176 (Varian, USA), the photometer detector output was recorded on Shimadzu Chromatopac C-R3A integrator and Varian recorder Model 9176. For spectrophotometric data acquisition and analysis Milichrom software and ''MultiChrom for Windows'' program (Ampersend, Moscow, Russia) were used. For pH measurements an Alkalit indicator (pH 6.0–10.0) (Merck, Darmstadt, Germany), a universal indicator (Brno, Czechia) and Digital pH Meter pH 525 (Wissenschaflich-Technische Werkstatten, Germany)

were applied. The centrifuge Model CLN 12 (Nauchpribor, Lvov, Ukraine), microcentrifuge Eppendorf 1540 (Eppendorf, Germany), vibrating mixer IKA Vibro Fix (IKA, Germany), and water multi-block heater (Lab-Line Instruments, IL, USA) were used during the sample preparation.

2.3. Preparation of serum and plasma samples

The blood (4.5 ml) was obtained from overnight fasting healthy laboratory personnel and cardiology reanimation patients by venipuncture and then drawn into vacutubes containing 0.5 ml 3.8% (w/w) sodium citrate in water, immediately centrifuged for 10 min at 3000 rpm (10 000 *g*) to obtain plasma, which was decanted, frozen in liquid nitrogen and stored at –70°C.

2.4. Determination of total homocysteine, cysteine and glutathione in plasma and serum

A 0.2-ml volume of thawed plasma (or serum) was pipetted into a 1.4-ml snap-cap polypropylene centrifuge vial, after addition of 0.075 ml of 427.2 m*M* triphenylphosphine, was capped, vigorously mixed and incubated for 10 min at 55°C. Trichloroacetic acid (10%, w/w, 0.15 ml) was added, the snapped caps were mixed, incubated for 10 min at 55°C and centrifuged at 10 000 *g* for 5 min. Aliquots of the supernatant solutions (0.02 ml) were pipetted into clean 1.4 ml polypropylene snap-cap centrifuge vials. Then 0.094 ml of 0.5 M sodium hydroxide was added to each vial, and after mixing 0.03 ml of 8.72 m*M* monobromobimane was pipetted. The samples were mixed and incubated for 5 min in darkness at room temperature. The vials were centrifuged at 10 000 *g* for 1 min and 0.05 ml of each sample was applied to both the reversed-phase HPLC systems. Sample vials were stored at –20°C in darkness.

2.5. Preparation of calibration standards

Stock solutions of 50 μmol/ml DL-*meso*-homocystine, 600 μmol/ml L-cystine, and 50 μmol/ml oxidized glutathione in 0.2 *M* hydrochloric acid were prepared. The working solutions were prepared by appropriate dilutions with 0.2 *M* hydrochloric acid as needed. For the preparation of calibration standards of human plasma, aliquots (200 μl) of plasma from a healthy donor were placed in a polypropylene microcentrifuge tubes (Eppendorf) and spiked with an appropriate amount of the working standard solution. Concentrations of exogenous ATs were 1.0, 2.0, 3.9, 7.8, 15.7, 31.3, 62.5, 125.0, 250.0, 375.0, 500.0, 1000.0 nmol/ml plasma for homocysteine; 1.0, 5.1, 10.2, 20.4, 40.7, 81.3, 122.0, 162.5, 244.3, 325.0, 497.5, 750.0, 1100.0 nmol/ml plasma for cysteine; and 1.0, 2.0, 3.9, 7.8, 15.7, 31.3, 62.5, 125.0, 250.0, 375.0, 500.0, 1000.0 nmol/ml plasma for reduced glutathione, assuming 100% disulfide reduction. Aliquots of the calibration standards were applied to determine the total homocysteine, cysteine, and glutathione contents as described in Section 2.5.

2.6. Chromatography

2.6.1. System 1 (see Section 2.2)

Samples were injected using an autosampler into a narrow-bore 75×2 mm column packed with 5-μm particles of Nucleosil C_{18}, pore size 120 Å (Machery–Nagel, Germany). The temperature was 35°C, detector dual wavelengths were 210 nm and 250 nm for monobimane-derivatized GSH analysis, and 250 nm for routine analyses of other derivatized thiol standards. The elution gradient profile was as follows:

- Profile 1.1: 0 μl–5% B; 1600 μl–13% B; 2800 μl–45% B; the flow-rate was 80 μl/min.
- Profile 1.2: 0 μl–6% B; 900 μl–11% B; 1820 μl–12% B; 2800 μl–50% B; the flow-rate was 75 μl/min.
- Elution solvent A was 0.1% TFA (pH 2.2) in water, solvent B was acetonitrile.

2.6.2. System 2 (see Section 2.2)

Samples were injected manually into the Model 210 injector loop by Hamilton syringe (Hamilton, Reno, NV, USA). The column (250×2 mm) packed with 5-μm particles of Ultrasphere ODS (Beckman Instruments) was used. The column temperature was 25°C, the flow-rate was 0.4 ml/min; and the photometer detector wavelength was 250 nm. The fluorescence detector operated with an exiting wavelength of 300 nm and an emission wavelength of 470 nm. The elution gradient profile was as follows:

- Profile 2.1: 0 min–10% B; 0–30 min–10–15% B;

30–40 min–15–100% B; 45–50 min–100–10% B.

- Elution solvent A was 0.1% TFA in water (pH 2.2), solvent B was 0.1% TFA in acetonitrile.

2.7. Statistics

Standard curves for plasma homocysteine, cysteine, and glutathione were constructed by adding the known amounts of DL-*meso*-homocystine, L-cystine, or oxidized glutathione, respectively, to normal plasma followed by the assay as described in Sections 2.5 and 2.6. The added DL-*meso*-homocystine and GSSG ranged from 0.5 to 500 nmol/ml and L-cystine from 0.5 to 550 nmol/ml. The absolute peak areas were plotted vs. the analyte concentration and the curves were fitted by least-squares linear regression analysis. A within-run precision for the method was obtained by injecting the same sample for three times consequently to both the HPLC systems and comparing the peak areas for cysteine, homocysteine, and glutathione derivatives obtained for three injections. A between-run precision was determined by derivatizing the same sample for three different times and injecting the resulted three derivative mixtures independently to both the HPLC systems. Data from recovery and precision studies are presented as mean±SD.

3. Results

The total plasma homocysteine, cysteine, and glutathione contents were determined in human plasma (or serum) in a few steps: (a) reduction of disulfides with triphenylphosphine; (b) deproteinization with trichloroacetic acid; (c) derivatization of reduced aminothiols with monobromobimane; and (d) separation of aminothiol–monobimane conjugates by reversed-phase HPLC with photometric and fluorescence detection. Resolution and detection of homocysteine, cysteine, and glutathione on gradient RP-HPLC System 1 are shown in Fig. 1 and in human plasma on System 2 in Figs. 4–7. Under the conditions described, the homocysteine–monobimane conjugate has a retention time of 16.7 min in System 1 and 18.5 min in System 2; the cysteine–monobimane, mB derivative of the major plasma

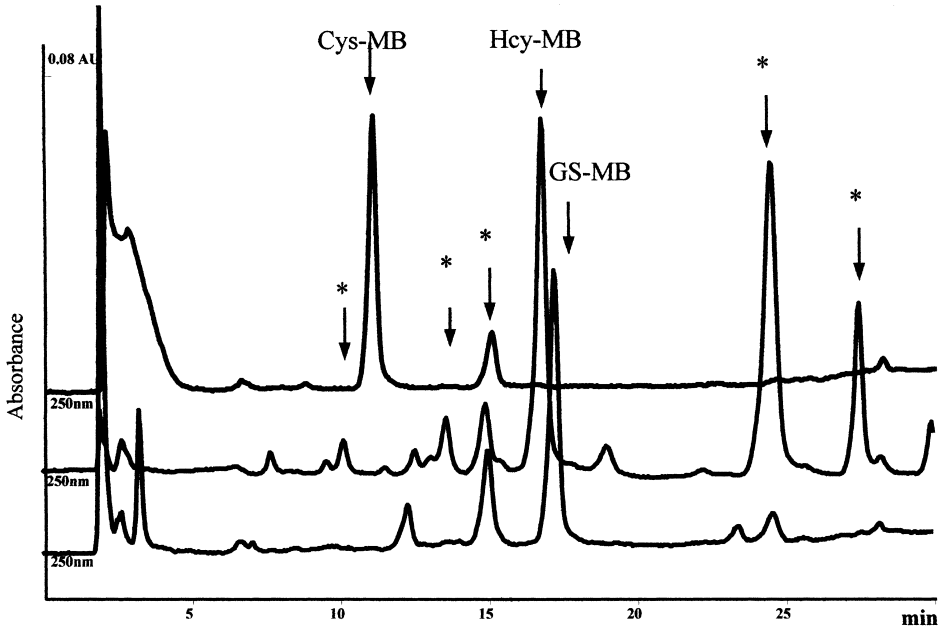

Fig. 1. RP-HPLC identification of monobimane conjugates of standard thiols (Cys, 2.33 nmol, Hcy, 3.12 nmol and GSH, 2.33 nmol) on the narrow-bore System 1 with the elution gradient profile 1.1. Monobimane hydrolysis products are marked by asterisks.

low-molecular-mass aminothiol, eluting with a retention time of 11.2 min in System 1 and 7.8 min in System 2; and glutathione–monobimane 17.6 min and 19.6 min, respectively. The retention times as well as the concentrations of these aminothiol–monobimanes derived from serum instead of plasma was identical (data not shown). We used two detection types for the method optimization and its application in human plasma analysis. HPLC System 1 was preferably used for the method optimization, routine thiol standard analysis, then employed to rough plasma assay. The detection wavelength of 250 nm was chosen as the local extinction maximum of the thiol–monobimane conjugates. Higher selectivity and sensitivity of the HPLC System 2 equipped with a fluorometer promoted to quantify AT–mB conjugates more precisely and reliable in a wider AT concentration range. It was used for routine qualitative and quantitative plasma aminothiol assays.

Standard curves for plasma homocysteine, cysteine and glutathione were constructed by adding the known amounts of authentic DL-*meso*-homocystine, L-cystine or oxidized glutathione standards to normal plasma followed by immediate assay. The total concentration (μM) of the three ATs in plasma ($\pm$SD) obtained from nine healthy overnight fasting males was 223.12 ($\pm$28.92) for Cys, 12.26 ($\pm$2.14) for Hcy and 6.97 ($\pm$1.82) for GSH, and from six healthy overnight fasting females it was 212.76 ($\pm$31.94) for Cys, 10.56 ($\pm$2.13) for Hcy and 6.12 ($\pm$1.88) for GSH.

A within-run precision for the method was obtained by injecting the same sample for three times consequently to both the HPLC systems and comparing the peak areas for cysteine, homocysteine and glutathione derivatives obtained for three injections. The RSD obtained for the within-run precision was 4.59% for the Cys derivative peak, 4.63% for the Hcy derivative and 4.86% for the GSH derivative. A between-run precision was determined by derivatizing the same sample for three different times and injecting three resulted mixtures independently to both the HPLC systems. A satisfactory analytical precision (4.86% RSD) was obtained without inclusion of an internal standard. The sensitivity of the method allows the detection of AT quantities >2–5 pmol.

4. Discussion

This method was based on previously published assays designed to determine ATs in human plasma and urine using sodium borohydride [1,2,5] or tri-*n*-butylphosphine [11,18], or dithioerythritol (DTE) [2] as a reducing agent and mBrB [1,2,5] or *N*-(1-

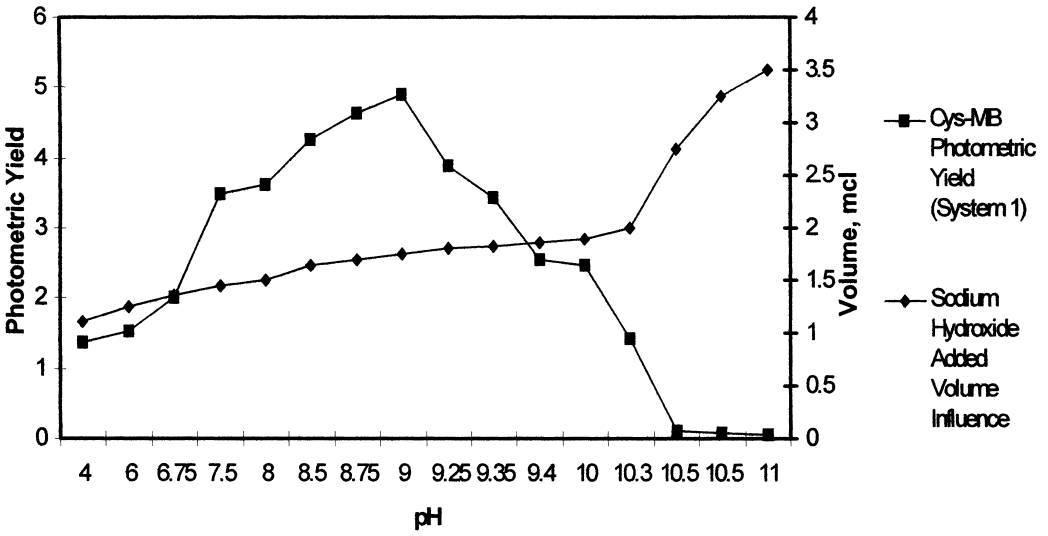

Fig. 2. The derivatization yield for cysteine in pH range 4–11.

pyrenyl)maleimide [7] as a derivatizing agent. We tried out to combine potent aspects of these assays, simplify the sample preparation step and develop efficient and common HPLC conditions with photometry and fluorimetry detection. According to Figs. 1–7, these modifications resulted in an acceptable separation of AT–monobimane conjugates providing correlation of quantitative data with the assay results published.

Determination of the total low-molecular-mass AT content in human plasma (or serum) requires the reduction of several disulfide species. It has been reported [2,17] that the reduced form of glutathione rapidly oxidizes (about 2.5 min) in human plasma to form GSSG disulfide. Cys and Hcy may oxidize even more rapidly than GSH, because the steric obstacle is smaller [3]. We used acidification and addition of EDTA to remove metal cations, routinely used to minimize oxidation. Among the reducing agents are sodium borohydride [1,2,5,20–22], 2-mercaptoethanol [19], DTE [2,22] and tri-*n*-butylphosphine [12,18], which could be used to cleave disul-

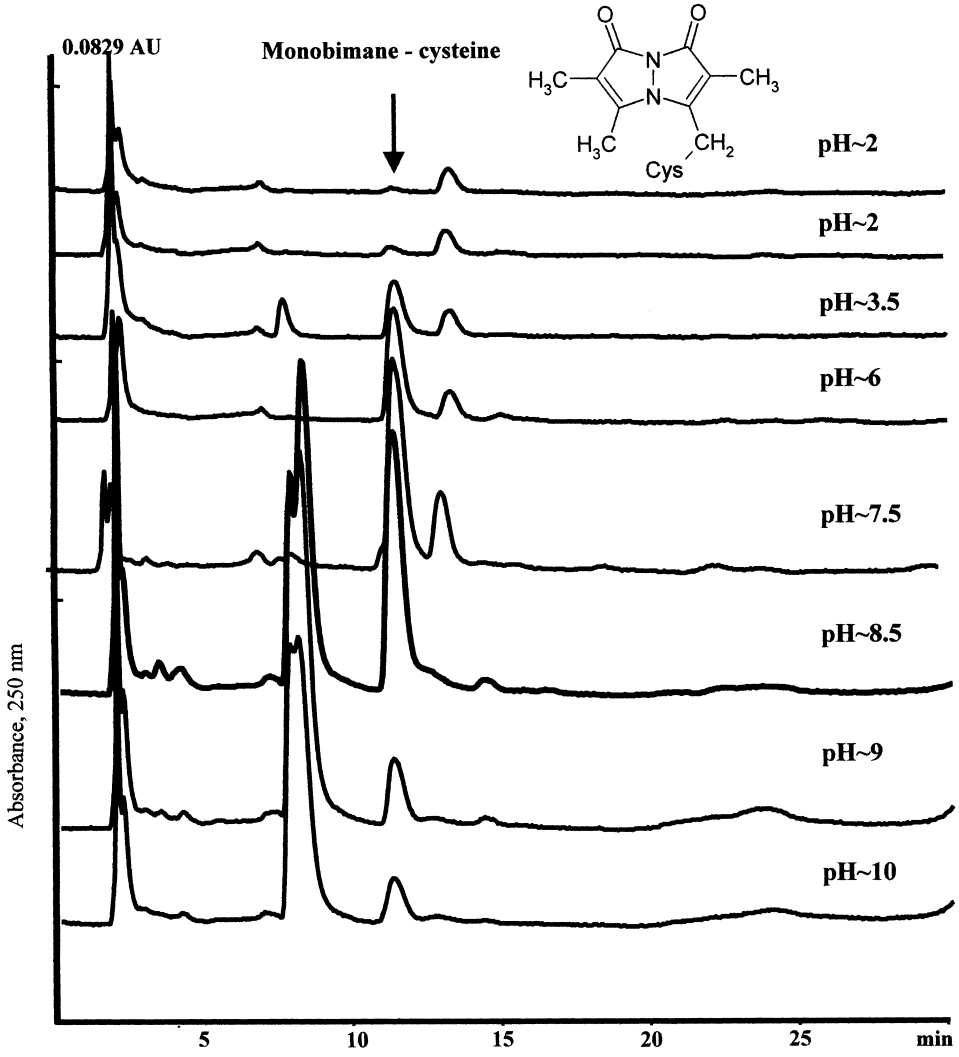

Fig. 3. Optimization of the monobimane derivatization of cysteine (~2.4 nmol) on the reaction pH (HPLC System 1, the elution gradient profile −1.2).

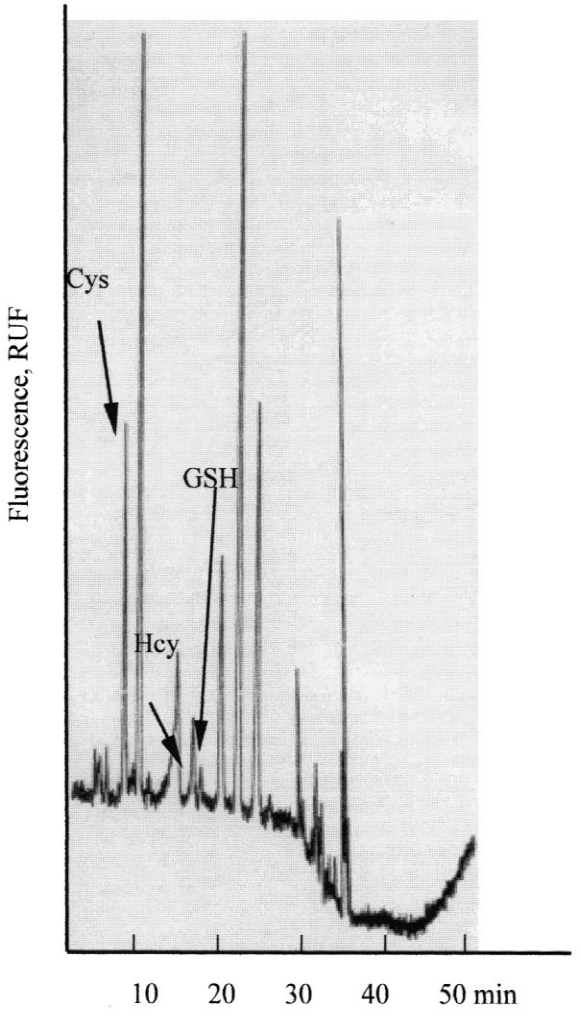

Fig. 4. RP-HPLC aminothiol identification in healthy human plasma with fluorescence detection. The aminothiol content is 206.7 nmol/ml for cysteine, 14.3 nmol/ml for homocysteine and 6.6 nmol/ml for glutathione.

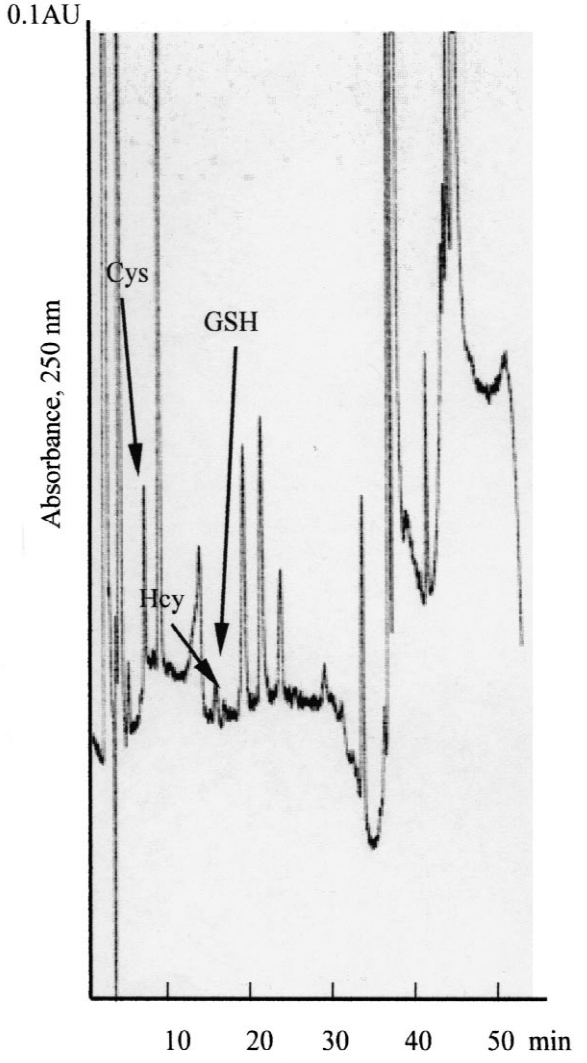

Fig. 5. RP-HPLC aminothiol identification in healthy human plasma with photometric detection. Concentrations as in Fig. 4.

fide bonds and release these thiols from plasma protein. Electrolytic reduction was also employed [7,10,12]. Such thiols as DTE or 2-mercaptoethanol could not improve the assay because of their reaction with mBrB leading to the formation of fluorescent compounds, interfering with the identification of the compounds to be studied, thus the chromatography separation could become more unfavorable and the analysis more complicated. Sodium borohydride is unstable in aqueous solution, a rather drastic reducing agent, and awkward mainly, because of foaming. Reduction by tri-*n*-butylphosphine is overshadowed by being explosion-proof at a high concentration. Triphenylphosphine is more convenient due to its high reactivity and greater safety. We selected this reducing agent and conditions for fast and complete reduction and thiol liberation. TP, used as a reducing agent, provided more reproducible and reliable results than sodium and potassium borohydrides (data not shown). The reduction step did not markedly prolong the overall analysis time. In addition to the reduction function, triphenylphosphine served as a

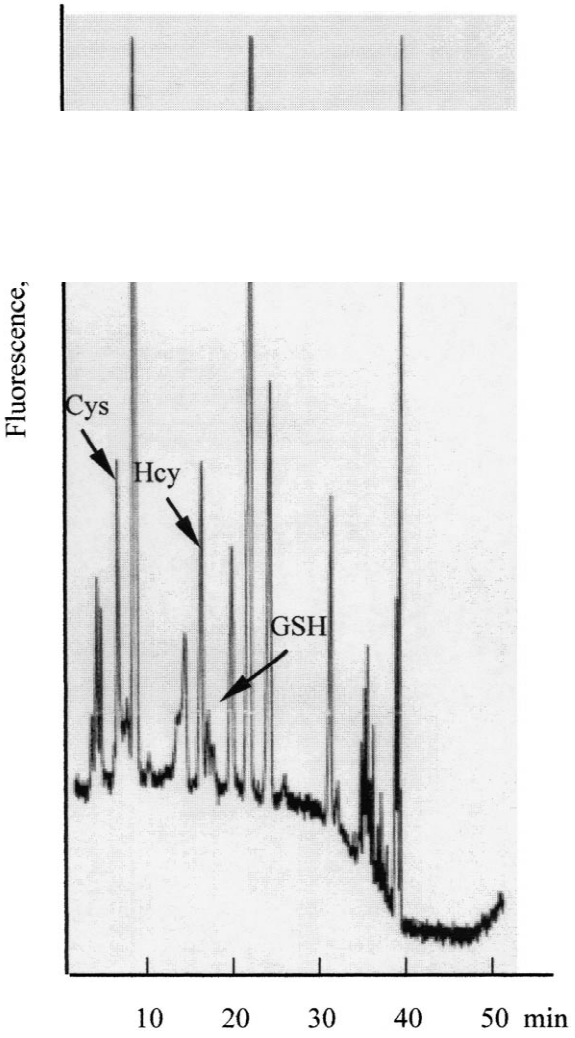

Fig. 6. RP-HPLC aminothiol identification in human plasma with fluorescence detection. The aminothiol content is 197.4 nmol/ml for cysteine, 164.6 nmol/ml for homocysteine and 7.9 nmol/ml for glutathione.

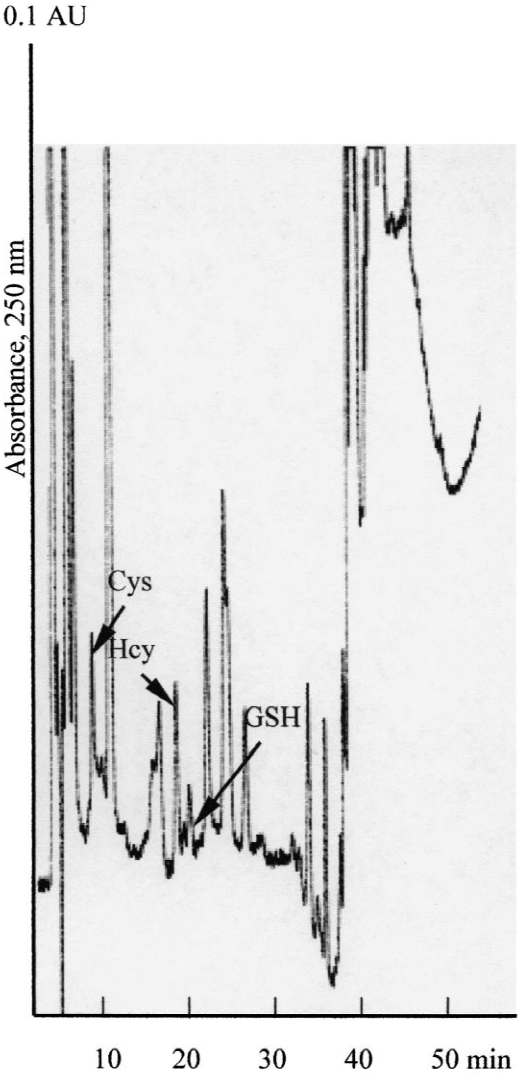

Fig. 7. RP-HPLC aminothiol identification in human plasma with photometric detection. Concentrations as in Fig. 6.

prohibitory agent for the re-oxidation of aminothiols before their derivatization. Moreover, to prevent this re-oxidation we employed an excess of triphenylphosphine. An addition of chlorohydroxic acid to triphenylphosphine solution sets conditions for catalyzing disulfide reduction [23].

Of various the thiol-specific derivatization reagents [1,2,5,7,11], we chose mBrB for the following reasons: the fluorescence intensity of the AT–monobimane (AT–mB) derivatives is sufficiently high to measure them in plasma at low concentrations; another advantage of mBrB as a derivatizing agent is its ability to react swiftly (for 2 min [1]) with free thiols at room temperature. Furthermore, considerable extinction of the AT–mB [24] permits us to optimize AT standards reduction and derivatization procedures and to perform rough plasma assay (data not shown) with the qualitative and quantitative AT–mB determination on HPLC System 1 equipped with the UV detector. Monobromobimane is photolabile,

unstable in water and at room temperature [1–4]. But it was relatively stable in water–acetonitrile solutions when stored at –20°C in darkness. Even under optimal conditions, multiple fluorescent peaks were formed. The mBrB solution should be changed after a few days of storage. Routinely, we prepared fresh mBrB solution before the analysis.

It is well known that mBrB spontaneously forms conjugates with thiols under physiological conditions [2,24]. When increasing the pH of the derivatization reaction by adding sodium hydroxide, the maximum yield of the monobimane–thiol derivatives was near pH 8.5 (Figs. 2 and 3). The optimal pH of the reaction medium was performed by alkalization. Such buffers as trimethylamine, sodium borate, Tris, as well as sodium hydroxide were used. The latter provided the best derivatization yield with the minimum sample dilution. Thus we adjusted sample pH before derivatization to ~8.5 by adding 0.094 ml of 0.5 *M* sodium hydroxide. The optimal pH of derivatization allowed us to avoid superfluous consumption of mBrB reagent and to minimize the formation of fluorescent monobimane hydrolysis byproducts, interfering with the detection and separation of AT–monobimane conjugates. An increase of derivatization temperature resulted in an elevation of monobimane hydrolysis extent (data not shown).

The sensitivity of the developed method is favorably compared with that of other low-molecular-mass AT assays [2,7,8]. High chromatographic resolution, low noise, combination of dual detection types, linearity of the standard curves, as well as high fluorescence intensity of AT derivatives stipulated the assay accuracy. HPLC conditions are favorably compared with some assays in their simple performance, omitting the use of several column types and complicated mobile phase that need changing [1,2,5,11].

In conclusion, the present assay includes three stages in order to determine the reduced, oxidized and protein-bound homocysteine, cysteine and glutathione as the total amounts in human plasma. The oxidized and protein-bound ATs were converted to their reduced forms with the use of triphenylphosphine; following precolumn derivatisation of free sulfhydryls with mBrB; the aminothiol–monobimanes were separated and quantified by reversed-phase high-performance liquid chromatography with fluorescence and additional on-line photometry detection. Application of this HPLC approach with two detection types demonstrated the possibility of Hcy determination at pathological concentration levels in human plasma with high reliability with only use of common and widespread UV detection. The method was optimized in reduction and derivatization procedures to obtain thiol–monobimane derivative maximum yield.

The method reported has several advantages: (1) high reactivity of triphenylphosphine as a reducing agent; (2) high reactivity and selectivity monobimane derivatization toward thiols; (3) fluorescenceless of the reagent and high fluorescence yield of the derivatives; (4) low hydrolysis rate under optimized alkaline conditions of the derivatization reaction; (5) simple sample preparation procedure; (6) simple and prevalent mobile phase; (7) simultaneous determination of all the mentioned low-molecular-mass aminothiols during the procedure. The final advantage of the method is it's easy use and high reliability.

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